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Association of m1 and m2 muscarinic receptor proteins with asymmetric synapses in the primate cerebral cortex: Morphological evidence for cholinergic modulation of excitatory neurotransmission

(immunohistochemistry/cholinergic system/visual cortex/prefrontal cortex/cortical afferents)

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ABSTRACT **Muscarinic m1 receptors traditionally are considered to be postsynaptic to cholinergic fibers, while m2 receptors are largely presynaptic receptors associated with axons. We have examined the distribution of these receptor proteins in the monkey cerebral cortex and obtained results that are at odds with this expectation. Using immunohistochemistry with specific antibodies to recombinant m1 and m2 muscarinic receptor proteins, we have demonstrated that both m1 and m2 receptors are prominently associated with noncholinergic asymmetric synapses as well as with the symmetric synapses that characterize the cholinergic pathways in the neocortex. At asymmetric synapses, both m1 and m2 receptor immunoreactivity is observed postsynaptically within spines and dendrites; the m2 receptor is also found in presynaptic axon terminals which, in the visual cortex, resemble the parvicellular geniculocortical pathway. In addition, m2 labeling was also found in a subset of nonpyramidal neurons. These findings establish that the m2 receptor is located postsynaptically as well as presynaptically. The association of m1 and m2 receptors with asymmetric synapses in central pathways, which use excitatory amino acids as neurotransmitters, provides a morphological basis for cholinergic modulation of excitatory neurotransmission.**

Muscarinic receptors in the central nervous system have been implicated in normal learning and memory (1–3), arousal (4–6), and motor and sensory modulation (4, 5, 7) as well as in conditions such as normal aging, Alzheimer and Parkinson diseases, schizophrenia, and depression (8–11). Recently, the cloning of five different genes encoding structurally similar muscarinic receptor proteins (m1–m5) (12, 13) has led to production of subtype-specific antibodies against recombinant muscarinic receptor proteins (14, 15). These antibodies have enabled us to identify the neuronal elements containing particular receptors in the cerebral cortex at a level of resolution and specificity not possible in previous autoradiographic studies of these receptors (16, 17) or in previous immunohistochemical analyses using nonselective muscarinic antibodies (18). Our findings have revealed an unexpected synaptic association of m1 and m2 receptors with fiber systems utilizing excitatory transmitters in the primate cerebral cortex.

MATERIALS AND METHODS

Tissue and Fixation. The results of this study are based on light and electron microscopic analysis of frontal and occipital lobe tissue from three adult macaque monkeys (*Macaca mulatta*). The monkeys were anesthetized with an intravenous overdose of sodium pentobarbital (Nembutal) (100 mg/kg) and after initial saline perfusion, one monkey was perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and two monkeys were perfused with 4% paraformaldehyde/0.08% glutaraldehyde/0.2% picric acid in PB. After perfusion, the frontal and occipital lobes were dissected into coronal blocks and postfixed for an additional 2 hr in the fixative described above without glutaraldehyde and picric acid. After fixation, the blocks for light microscopy were immersed in increasing concentrations of sucrose in PB before sectioning on the freezing microtome. For electron microscopy, the tissue was washed in PB and cut on a Vibratome. In the frontal cortex, our analysis focused on prefrontal area 46 (19), while in the occipital lobe we concentrated on primary visual cortex (20).

Immunohistochemistry. The same immunohistochemical protocol was used for both light and electron microscopic immunohistochemistry. Triton X-100 was not used in the immunohistochemical procedure. To enhance the penetration of the antibody, sections for electron microscopy were immersed in 15% sucrose in PB and freeze–thawed with liquid nitrogen. After they were washed in phosphate-buffered saline (PBS, pH 7.4), the sections were preincubated in blocking serum containing 5% normal goat serum and 1% bovine serum albumin in PBS. This solution was used for all antibody dilutions. The sections were then incubated for 48 hr (4°C) with subtype-specific antibodies against recombinant m1 and m2 receptor proteins at a working dilution of 0.5 μg/ml. Production, characterization, and specificity of these affinity-purified polyclonal antibodies against recombinant proteins are described elsewhere (14, 15). The sections were further processed by the avidin-biotin method using goat anti-rabbit biotinylated antibodies (Vector Laboratories) and an avidin-biotin-peroxidase complex (ABC Elite kit; Vector Laboratories). The immunoreaction product was visualized by using 0.05% diaminobenzidine in the presence of 0.01% hydrogen peroxide in PB. For light microscopy, the sections were lightly treated with 0.01% osmium tetroxide or intensified with Giemsa solution. Adjacent immunoreacted sections were counterstained with cresyl violet for delineation of cortical layers. The sections were dehydrated in ethanol, cleared with xylene, coverslipped with Permount, examined, and photographed with a Zeiss Aristoplan microscope.

Sections for electron microscopy were postfixed in 2% osmium tetroxide in PB and flat embedded in Durcupan (Fluka) between liquid release pretreated glass slides and coverslips. Selected blocks were cut serially into ultrathin sections on an Ultramicrotome (Reichert). The ultrathin sections were stained with lead citrate and uranyl acetate or were left unstained and examined with a Philips CM-10 transmission electron microscope.

Two types of controls for the immunohistochemical procedure were performed. (*i*) Primary antibodies were omitted

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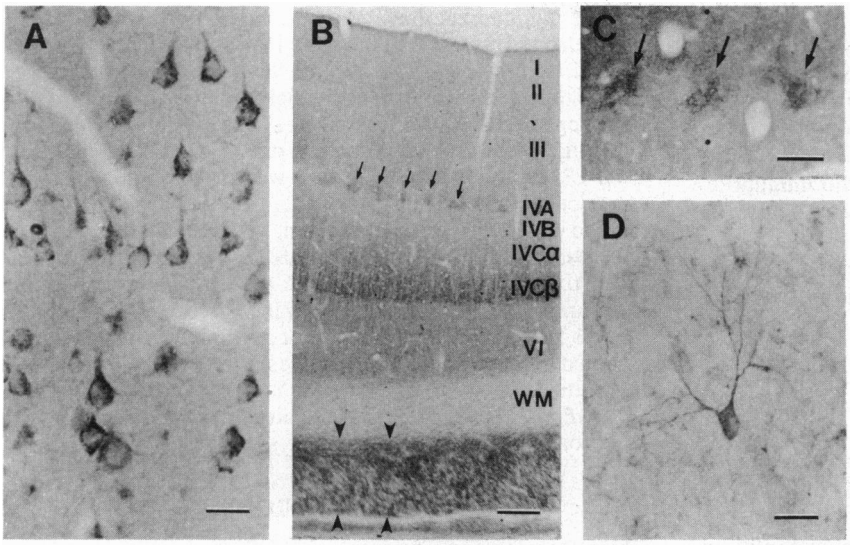

FIG. 1. (*A–D*) Light microscopic localization of m1 and m2 receptor immunoreactivity in the prefrontal and visual cortex in the macaque. (*A*) m1 receptor immunoreactive pyramidal neurons in layer III of the dorsal bank of the principal sulcus (area 46). Reaction product is visible in the proximal part of apical and basal dendrites as well as in cell bodies. (*B*) m2 receptor immunoreactivity in the primary visual cortex and subjacent white matter (WM). Immunoreactivity is prominent in layer IVA and deeper layer IVC as well as in the fiber bundles of geniculocortical radiation (between arrowheads). In layer IVA, the labeling pattern is patchy (arrows). (*C*) Higher magnification of the m2-positive patches (arrows) in layer IVA, which are formed by fibers and boutons. (*D*) Golgi-like staining of a nonpyramidal neuron labeled with the m2 receptor antibody in layer II of area 46. (Bars: *A* and *D*, 25 μm; *B*, 200 μm; *C*, 50 μm.)

from the immunohistochemical procedure. (*ii*) The m1 and m2 antibodies were preadsorbed 1 hr before the immunohistochemical reaction with fusion proteins (50 μg of the fusion protein was added to 800 μl of the diluted antibody). Antibody binding was successfully blocked with preadsorption procedures and no immunoreaction product was observed.

RESULTS

m1 Receptor Immunoreactivity. The m1 receptor protein was found exclusively in cell bodies and proximal dendrites of neurons in all cortical layers and adjacent white matter in both the prefrontal (area 46) and the primary visual cortex (Fig. 1*A*). Staining was most prominent in pyramidal neurons in layers III (Fig. 1*A*) and V/VI in the prefrontal cortex and in layers III and VI in the visual cortex. Electron microscopic analysis of labeled neurons revealed m1 receptors in the Golgi complex and endoplasmic reticulum as well as in the cytoplasm of large and small dendrites and dendritic spines (Fig. 2). This localization provides evidence of neuronal synthesis of the receptor protein and of its transport to distal processes.

Immunoreactivity denoting the m1 receptor was observed in postsynaptic elements of both symmetric and asymmetric synapses (Fig. 2) throughout the cortical depth. The asymmetric synapses were encounted more frequently and these were preferentially on dendritic spines (Fig. 2). In contrast, the immunoreactive symmetric synapses, which were less common, were predominantly observed on dendritic shafts. In addition to labeling postsynaptic densities, immunoreaction product was also found in the cytoplasm of labeled postsynaptic structures (Fig. 2). Serial section analysis of selected asymmetric synapses demonstrated labeled postsynaptic densities through three to five adjacent sections (Fig. 2 *B–E*).

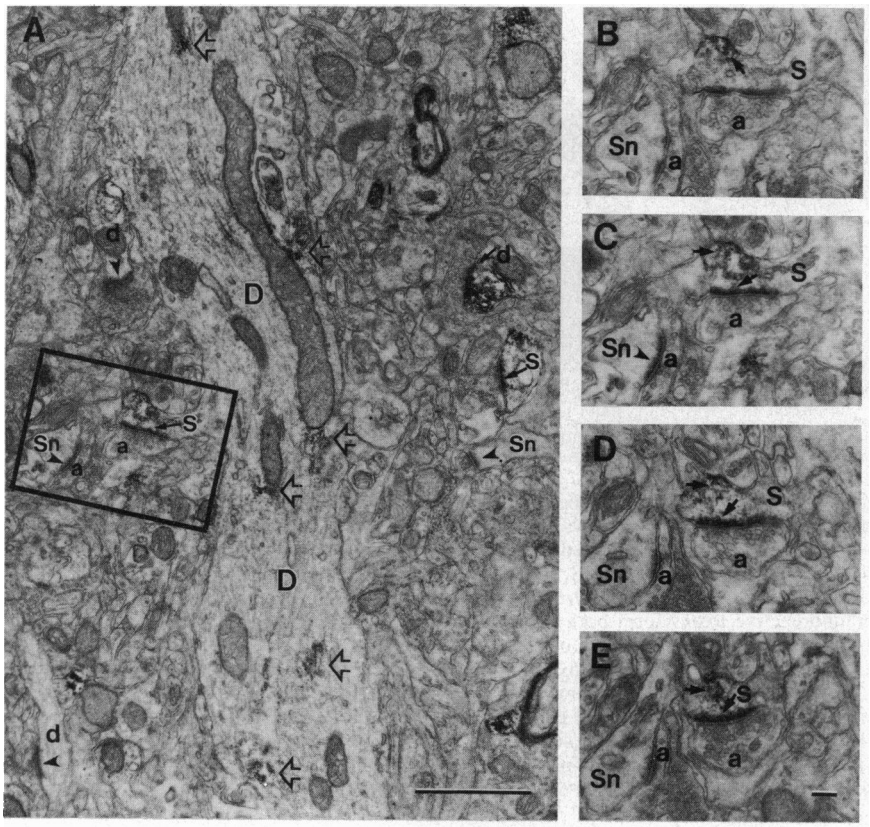

FIG. 2. (*A*) Electron micrograph of an m1 immunoreactive spiny dendrite (D) and surrounding neuropil from layer III in area 46. Reaction product is distributed throughout the cytoplasm (open arrows). In the boxed area, there are two asymmetric synapses—one formed on an m1 receptor immunoreactive spine (S with arrow) and one on an immunonegative spine (Sn with arrowhead). Framed area is enlarged in *C*. In the rest of the neuropil m1 receptor is associated with postsynaptic sites of asymmetric synapses formed by immunonegative axons (a), spine (S; arrow), and dendrite (d; arrow). Note the difference in staining intensity between immunopositive and immunonegative postsynaptic densities (Sn, d; arrowheads). (*B–E*) Serial sections through m1 receptor immunopositive (S) and immunonegative (Sn) dendritic spines from the boxed area in *A*. Both are targets of immunonegative axons (a), which form asymmetric synapses on them. In serial sections in *C–E*, immunoreaction product is enriched at both the postsynaptic densities and the adjacent cytoplasm (arrows), where, in the section shown in *B*, its association with a postsynaptic density is less conspicuous and more evident in the cytoplasm (arrow). Note the difference in staining intensity between m1 immunopositive (arrow) and immunonegative (arrowhead) postsynaptic densities in *C*. (Bars: *A*, 1 μm; *B–E*, 0.5 μm.)

m2 Receptor Protein Immunoreactivity. m2 immunoreactivity was localized predominantly in presynaptic axons (Figs. 1*C* and 3 *C–H*) and to a lesser extent postsynaptically in spines and small dendrites (Fig. 3 *A* and *B*); m2 immunoreactivity was also observed in a population of nonpyramidal neurons in the cortex (Fig. 1*D*) and white matter. A deceptive feature of the presynaptic labeling was that the immunoreaction product was commonly located in the axoplasm at some distance from presynaptic sites (Fig. 3 *C–F*). In contrast, in m2 immunoreactive postsynaptic elements, immunoreaction product was always observed within the postsynaptic densities as well as in the cytoplasm (Fig. 3 *A* and *B*).

The m2 immunoreactivity was associated with asymmetric (Fig. 3 *A–F*) as well as symmetric (Fig. 3 *G* and *H*) synapses in both presynaptic and postsynaptic elements throughout the cortical depth of the prefrontal and visual cortex. As with the m1 receptor, m2 labeling was more frequently associated with asymmetric synapses. Labeled asymmetric synapses were preferentially formed on dendritic spines (Fig. 3 *A–C*, *E* and *F*), while symmetric synapses were predominantly on dendritic shafts (Fig. 3 *G* and *H*).

The immunoreactive axons formed dense bands in layer V in prefrontal area 46 and in layers IVA and IVC in the primary visual cortex (Fig. 1*B*). In both the cortex and subcortical white matter (Fig. 1*B*), the m2 labeling in the visual cortex had the appearance of geniculocortical parvicellular projections; labeling in layer IVA was patchy and discontinuous (Fig. 1 *B* and *C*), while that in layer IVC was more homogenous and restricted to its deeper part—i.e., sublayer IVCβ (Fig. 1*B*). Based on the size and distribution, m2-labeled axonal patches in layer IVA appear to interdigitate with cone-shaped clusters of pyramidal neurons recently described by Peters and Sethares (21). Electron microscopic analysis of m2 labeling in layers IVA and IVC revealed that asymmetric synapses were formed by two types of immunopositive axonal profiles: small (<1 μm in diameter; Fig. 3*F*) and large (>1.5 μm in diameter; Fig. 3*E*), the latter with multiple mitochondria, round vesicles, and forming multiple asymmetric synapses.

DISCUSSION

The present study features three findings with respect to muscarinic receptor complexes in the primate cortex. The

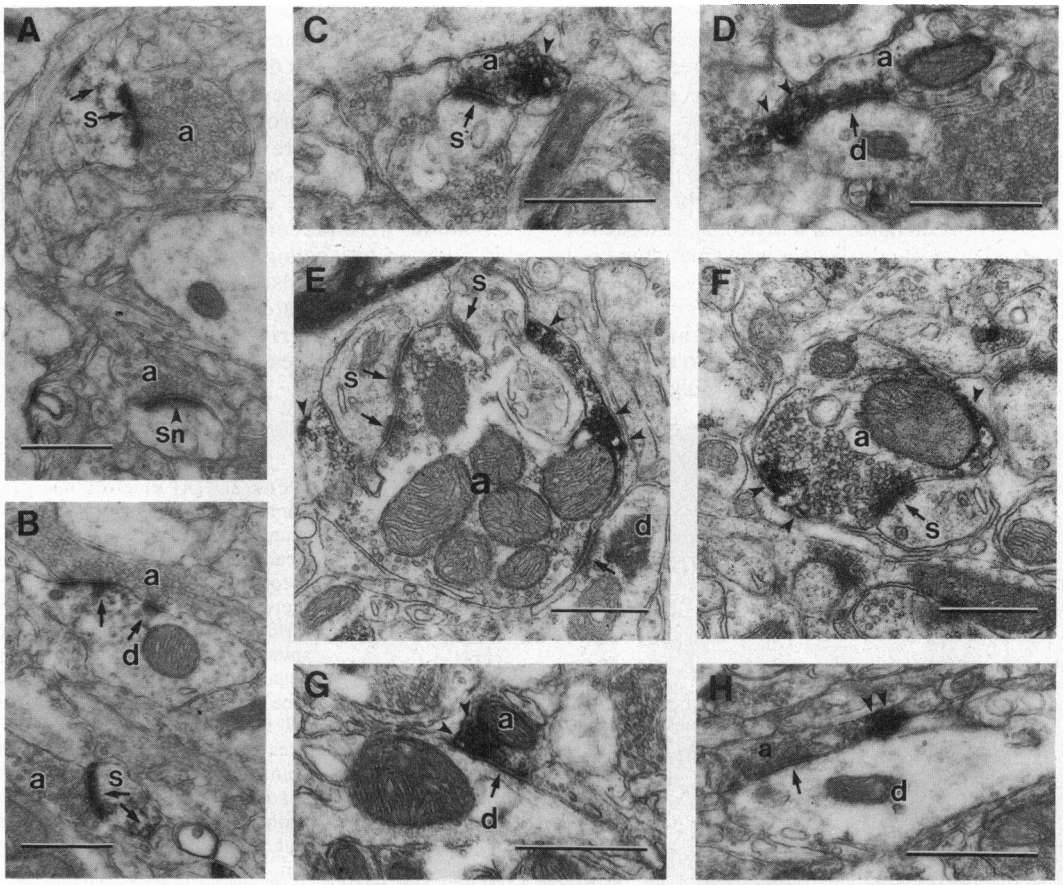

FIG. 3. Postsynaptic (*A* and *B*) and presynaptic (*C–H*) localization of m2 receptor immunoreactivity in prefrontal and visual cortex. (*A*) Unlabeled axons (a) form asymmetric synapses on m2 immunoreactive (S; arrows) and immunonegative (Sn; arrowhead) spine in layer III of prefrontal area 46. In the m2 receptor, labeled spine immunoreactivity is associated with both cytoplasm and postsynaptic density (arrows). (*B*) Unlabeled axons (a) in asymmetric synaptic contacts with immunopositive spine (S; arrows) and dendrite (d; arrows) in layer IVA of the primary visual cortex. Note that postsynaptic m2 receptor labeling in spines and small dendrites has the same appearance as m1 receptor labeling in these structures (compare with Fig. 2). m2 receptor immunoreactive axons form both asymmetric (*C–F*) and symmetric (*G* and *H*) synapses in prefrontal and visual cortex. Reaction product is usually associated with portions of axoplasm (arrowheads) at a distance from the presynaptic sites (*C*, *E*, *F*, and *H*). (*C* and *D*) Electron micrographs of two immunoreactive axons (a) in the superficial part of layer III in area 46, which form clear asymmetric synapses with a spine (s) (*C*) and a dendrite (d) (*D*). Arrows point to prominent postsynaptic densities. (*E* and *F*) m2 immunoreactive axons (a) in layer IV of the primary visual cortex. (*E*) "Baseball glove"-shaped immunoreactive large axonal profile (>2 μm in diameter) forms multiple asymmetric synapses (arrows) with spines (s) and a dendrite (d) in one of the patches of layer IVA. (*F*) Smaller diameter immunoreactive axonal profile forms an asymmetric synapse with a spine in layer IVC. (*G* and *H*) Immunoreactive axons (a) form symmetric synapses with dendritic shafts in layer II of area 46. Note parallel, equidense pre- and postsynaptic densities characteristic of symmetric synapses (arrows). (Bars = 0.5 μm).

first is the association of both m1 and m2 receptors with asymmetric as well as symmetric synapses. Labeled asymmetric synapses were more frequent than symmetric ones and were found predominantly on dendritic spines, whereas m1 and m2 symmetric synapses were formed primarily on dendritic shafts. Second, contrary to common belief, the presence of m2 receptor protein in spines and dendrites establishes that this receptor is not exclusively presynaptic but is also found postsynaptically on pyramidal and nonpyramidal neurons. Finally, the m2 receptor is present in axons that form asymmetric synapses as well as in axons forming the symmetric synapses characteristic of the cholinergic afferent system in the cortex. All three findings suggest an important role of muscarinic receptors in the modulation of noncholinergic transmission in the primate cortex.

Specificity of Immunohistochemical Reaction. Given the unexpected result of a strong association of muscarinic receptors with asymmetric noncholinergic synapses, the question of antibody specificity should be examined. The immunohistochemical staining for m1 and m2 receptor proteins appeared specific by all common criteria used to demonstrate antibody specificity. The immunostaining was completely blocked by preadsorption with the fusion proteins and was very selective with respect to neuronal elements on low background staining. In addition, the affinity-purified m1 and m2 antibodies have been raised against fusion proteins that encompass parts of the nonconserved third cytoplasmic loop (i3) of muscarinic receptors—i.e., they are subtype specific (14, 15). Furthermore, each antibody bound a single cloned receptor subtype by immunoprecipitation. Finally, the distribution of m1 and m2 receptor determined by immunoprecipitation is similar to that observed by immunohistochemistry (14, 15). Nevertheless, as is the case with almost all immunohistochemistry, we cannot exclude the possibility that our antibodies recognize some other proteins that have very high sequence homology.

Cholinergic Synaptic Architecture and Muscarinic Receptors. The present study confirms the relationship of m1 and m2 receptors to the cholinergic innervation of the cerebral cortex. The m1 and m2 receptors are found in postsynaptic elements apposed to symmetric synapses, which are characteristic for the cholinergic innervation shown in both primates (22) and cats (23–25). Furthermore, these synapses are found predominantly on dendritic shafts, which are the major postsynaptic target of cholinergic axons (22–25). In addition, the m2 receptors are observed in the axons that form symmetric synapses and are presumably the well-established autoreceptors on cholinergic axons. Monoaminergic axons also form symmetric synapses in the cortex (26) and it might be argued that they also bear m2 receptors. This possibility seems unlikely given that the m2-positive axons in the present study have few of the ultrastructural features of monoaminergic axons (also see below).

Noncholinergic Circuitry Involving m1 and m2 Receptors. The present evidence that m1 and m2 receptors are found in pre- or postsynaptic elements of asymmetric synapses provides evidence that these muscarinic receptors are associated with fiber systems other than cholinergic. The fact that these asymmetric synapses are predominantly formed on dendritic spines also supports this conclusion, since only a small proportion of cholinergic synapses are found on dendritic spines (22, 24, 25). The obvious candidates for the presynaptic component of m1 and m2 postsynaptic sites in asymmetric synapses are axons of the corticocortical and thalamocortical pathways. Both of these systems form asymmetric synapses and their postsynaptic targets are predominantly dendritic spines (27, 28), where the majority of postsynaptic m1 and m2 receptors are localized. Moreover, the finding that asymmetric synapses in the prefrontal and visual cortex are present in all layers and not only in layer IV, the major site of the thalamocortical innervation (27, 29–31), also speaks for involvement of corticocortical axons, which terminate in all cortical layers (32, 33). However, in the primary visual cortex, we suppose that the presynaptic elements in m1- and m2-positive synapses are associational fibers because the primary visual cortex does not receive commissural projection except at its border with visual association cortex (34).

Some thalamic and corticocortical axons that form asymmetric synapses with dendritic spines are likely to be bearers of m2 receptors. Although monoaminergic axons can form asymmetric synapses in the primate cortex (ref. 35; J. Smiley and P.S.G.-R., unpublished data), this source of afferents can essentially be excluded because the m2-positive axonal boutons observed in this study were devoid of the dense core vesicles characteristic of cortical monoaminergic axons. Moreover, in the visual cortex, the m2-bearing axons resemble geniculocortical axons originating in the parvicellular laminae of the lateral geniculate body, which are likely bearers of m2 receptors. As revealed by tracing experiments in monkeys, these geniculocortical projections exhibit a patchy distribution in layer IVA and a more continuous band in layer IVCβ (29, 30, 36), corresponding exactly to the pattern of m2 receptor labeling reported here. Also, the large m2 receptor immunoreactive axonal boutons with numerous mitochondria and multiple synaptic contacts in layers IVA and IVC closely resemble the ultrastructural features characteristic of geniculocortical synaptic morphology as revealed by degeneration and tracing electron microscopic studies (28, 37). In addition, axon bundles in the trajectory of the geniculocortical radiation (Fig. 1*B*) and in parvicellular laminae of the lateral geniculate body (unpublished data) also exhibit m2 immunoreactivity. The association of m2 receptors with thalamocortical axons is also supported by recent studies showing that m2 binding sites in cingulate cortex are diminished after lesions of the anterior thalamic nucleus (38, 39). Neurons in this thalamic nucleus have also been shown to possess mRNA for m2 receptors (40).

Not all thalamocortical systems bear m2 receptors, however. For example, the present analysis shows specificity even within the same thalamocortical system, since only the parvicellular geniculocortical axons and not those of the magnocellular fiber system appear to exhibit m2 labeling. In prefrontal cortex, the m2 receptor immunoreactive boutons, which form asymmetric synapses in supra- and infragranular layers, may belong to corticocortical axons.

Excitatory Amino Acids/Muscarinic Interactions in Cerebral Cortex. Since it is generally accepted that excitatory amino acids like glutamate and aspartate are the neurotransmitters released at the asymmetric synapses of thalamic and corticocortical projections (41–43), the present study offers evidence for cholinergic modulation of excitatory neurotransmission via m1 and m2 receptors. Acetylcholine may modulate excitatory neurotransmission either postsynaptically acting through m1 and m2 receptors on dendritic spines or presynaptically via m2 receptors on axons, which form asymmetric synapses with dendritic spines. Because the majority of spines belong to pyramidal neurons, we can assume that cholinergic modulation at spines predominantly influences pyramidal neurons, possibly also spiny stellate neurons in layer IV of visual cortex. Since these receptors are not postsynaptic to cholinergic axons, our findings raise the possibility that acetylcholine may influence m1 and m2 receptors at asymmetric junctions by diffusion from neighboring cholinergic axons. Such nonjunctional transmitter release has already been suggested for several neurotransmitter systems (44). Indeed, analysis of cholinergic synapses in the primate neocortex (22) has revealed many cholinergic boutons in close nonjunctional appositions with dendritic spines receiving asymmetric input. In the cat visual cortex, cholinergic axons are often juxtaposed to asymmetric axos-

pinous contacts, some of which exhibit presynaptic glutamate immunoreactivity (25). The mechanism of muscarinic modulation in the primate cerebral cortex may be similar to the glycinergic mechanisms in the retina, where presumed glutamatergic photoreceptor synapses can be immunostained for the glycine receptor but not for glycine. These studies suggest that glycine diffuses to these receptors from other sources in the retina, possibly from glycinergic interplexiform cells (45).

Anatomical Substrate for Physiological Responses. The present results offer a morphological basis for a number of physiological observations in the neocortex and hippocampus. Presynaptically, acetylcholine inhibits excitatory input via muscarinic receptors, leading to disfacilitation (7). Pharmacological analysis in hippocampus has shown that acetylcholine inhibits the release of both glutamate and aspartate via m2 receptors (46, 47). These mechanisms may be achieved through the m2 receptors shown here on axons forming asymmetric synapses. In the macaque visual cortex, layers IVA and IVC, which are rich in m2 receptor immunoreactive axons, also receive a strong cholinergic input (48). These cholinergic fibers may be the source of acetylcholine for m2 receptor targets on thalamocortical fibers.

Postsynaptic modulation may involve both m1 and m2 receptors. Acetylcholine reduces potassium currents in cortical pyramidal neurons, which leads to their increased excitability to incoming thalamocortical and corticocortical input (5). Specifically, acetylcholine facilitates responses of sensory cortical neurons to stimulation of their receptive fields through muscarinic receptors (5, 7). In the hippocampus, acting via muscarinic receptors, acetylcholine has a long-lasting facilitatory effect on excitatory postsynaptic potentials mediated through *N*-methyl-D-aspartate receptors (49). Thus, postsynaptic modulation of excitatory neurotransmission might be mediated through m1 or m2 receptors on the spines of pyramidal neurons.

m2 receptor protein was also found postsynaptically in certain populations of nonpyramidal neurons in the cortex and white matter. Preliminary light and electron microscopic analysis of these neurons shows that they all have characteristics of interneurons. Since interneurons in the neocortex are predominantly inhibitory, utilizing γ-aminobutyric acid as a neurotransmitter, our data may serve as morphological substrate for the physiological action of acetylcholine on cortical interneurons. Physiological studies have suggested that cholinergic excitation of interneurons is mediated via m2 receptors (50). However, this mechanism of cholinergic modulation may apply to only a certain population of interneurons, because not all nonpyramidal neurons in the cortical areas examined bear m2 receptor immunoreactivity.

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